Quantitative Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization–Mass Spectrometry

Wm. Craig Byrdwell*, Edward A. Emken, William E. Neff, and Richard O. Adlof

FQS, NCAUR, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: Atmospheric pressure chemical ionization–mass spectrometry (APCI–MS) was used for quantitative analysis of triglycerides (TG) separated by reverse-phase high-performance liquid chromatography. APCI–MS was used for analysis of mono-acid TG standards containing deuterated internal standard, of a synthetic mixture of heterogeneous TG, of randomized and normal soybean oils and of randomized and normal lard samples. Quantitation of the TG by four approaches based on APCI–MS were compared, and these were compared to quantitation obtained using liquid chromatography (LC) with flame-ionization detection (FID). The APCI–MS methods were based on (i) calibration curves from data for mono-acid TG standards, (ii) response factors obtained from a synthetic mixture of TG, (iii) response factors calculated from comparison of randomized samples to their statistically expected compositions, and (iv) response factors calculated from comparison of fatty acid (FA) compositions calculated from TG compositions to FA compositions obtained by calibrated gas chromatography (GC) with FID. Response factors derived from a synthetic mixture were not widely applicable to samples of disparate composition. The TG compositions obtained using APCI–MS data without application of response factors had average relative errors very similar to those obtained using LC–FID. Numerous TG species were identified using LC/APCI–MS which were undetected using LC–FID. Two quantitation methods, based on response factors calculated from randomized samples or on response factors calculated from FA compositions, both gave similar results for all samples. The TG compositions obtained using response factors calculated from FA compositions showed less average relative error than was obtained from LC–FID data, and were in good agreement with predicted compositions for the synthetic mixture and for randomized soybean oil and lard samples.

Lipids 31, 919–935 (1996).

Early (1) and recent (2) reviews have summarized the use of several types of two-dimensional detection methods for analysis of triglycerides (TG) using high-performance liquid chromatography (HPLC). These methods have included refractive index, ultraviolet-vis spectrophotometric, moving belt flame-ionization (FID), and evaporative light-scattering (ELSD) detection. While each of these detection methods has its strengths and weaknesses with regard to solvent compatibility, sensitivity, and other factors, the accuracy of the quantitative data of all these methods is dependent on the quality of the chromatographic resolution of the TG species. In complex mixtures which contain numerous species with the same equivalent carbon numbers, complete, or even partial, resolution of chromatographically overlapped species may not be possible. To solve this problem several types of mass spectrometric detection have been successfully applied to the identification and quantitation of molecular species of TG mixtures. Several interface types, such as fast atom bombardment, thermospray, direct inlet, and electrospray have been used, and these applications have been recently reviewed (3). One of the simplest, yet effective, ionization interfaces for use in a chromatographic system has been the direct inlet interface.

We have reported results that demonstrated that atmospheric pressure chemical ionization (APCI) is similarly a simple, versatile ionization source for analysis of TG. Our recent publications (4,5) described the application of APCI–mass spectrometry (MS) to the qualitative analysis of simple homogeneous (mono-acid) TG and genetically modified soybean oils (SBO) separated using reverse-phase HPLC (RP-HPLC). We showed that the mass spectra of TG obtained using APCI–MS were relatively simple and consisted primarily of diglyceride (DG) ions, $[M - RCOO]^+$ or $[DG]^+$, and protonated molecular ions, $[M + 1]^+$. The degree of unsaturation was the primary factor in determining the amount of DG vs. TG ions formed. The mass spectra of TG containing more than four sites of unsaturation contained protonated TG ions as base peaks. DG fragments were the base peaks in spectra of TG containing less than three sites of unsaturation. Mass spectra of TG with three or four sites of unsaturation contained either DG fragments or protonated TG ions as base peaks, depending on the fatty acid (FA) distribution within the TG. No TG protonated molecular ion (only DG) was observable from TG containing only saturated FA. Thus, the utility of APCI–MS for qualitative analysis of TG has been well demonstrated, but the potential for quantitative analysis using this ionization technique has not been explored.

*To whom correspondence should be addressed at FQS, NCAUR, USDA, ARS, 1815 N. University St., Peoria, IL 61604.

Abbreviations: ACN, acetonitrile; APCI–MS, atmospheric pressure chemical ionization–mass spectrometry; DG, diglyceride; EIC, extracted ion chromatogram; ELSD, evaporative light-scattering detector; FA, fatty acid; FAME, fatty acid methyl esters; FID, flame-ionization detector; GC, gas chromatography; L, linoleic acid (18:2); LC, liquid chromatography; Ln, linolenic acid (18:3); M, myristic acid (14:0); O, oleic acid (18:1); P, palmitic acid (16:0); Po, palmitoleic acid (16:1); RP-HPLC, reverse-phase high-performance liquid chromatography; S, stearic acid (18:0); SBO, soybean oil; TG, triglyceride.

Reported here is the extension of APCI–MS to the quantitative analysis of several model and natural TG samples. We report the quantitative analysis of a synthetic mixture containing homogeneous (mono-acid) TG with d_{12}-tripalmitin as internal standard, of a synthetic mixture of thirty-five TG with randomly distributed FA, of randomized and normal SBO and of randomized and normal lard samples. Comparison of quantitative results based on several approaches is presented: (i) construction of calibration curves from mono-acid TG standards, (ii) response factors obtained from a synthetic mixture of heterogeneous (mixed-acid) TG, (iii) response factors calculated from the comparison of randomized samples to their statistically expected compositions, and (iv) response factors calculated from comparison of compositions calculated from TG compositions to compositions obtained by calibrated gas chromatography (GC) with FID detection.

MATERIALS AND METHODS

TG. TG standard mixture "HPLC #G-2" was obtained from Nu-Chek-Prep (Elysian, MN). The TG standard mixture contained equal weights of the following mono-acid TG: tripalmitin (PPP), tripalmitolein (PoPoPo), tristearin (SSS), triolein (OOO), trilinolein (LLL), and trilinolenin (LnLnLn), where P is palmitic acid, S is stearic acid, O is oleic acid, L is linoleic acid, and Ln is linolenic acid. A stock solution of "HPLC #G-2" was used to prepare solutions containing: 30 μg/μL, 15 μg/μL, 6 μg/μL, 3 μg/μL, 0.6 μg/mL, 0.3 μg/μL, and 0.06 μg/μL total TG. This resulted in solutions containing the following amounts of individual TG standards: 5 μg/μL, 2.5 μg/μL, 1.0 μg/μL, 0.5 μg/μL, 0.1 μg/μL, 0.05 μg/μL, and 0.01 μg/μL. d_{12}-PPP was added to each standard solution at a concentration of 1.01 μg/μL as internal standard. The d_{12}-PPP was prepared in our laboratory by reduction of methyl *cis*-9-hexadecenoate-13,13,14,14-d_4 [prepared in a similar manner as the methyl 9-*cis*-octadecenoate analogue (6)] with Wilkinson's Catalyst (7) and hydrogen to the saturated fatty acid methyl ester (FAME), methyl palmitate-d_4. The FAME was converted to the FA with alcoholic KOH and reacted with glycerol and *p*-toluenesulfonic acid to yield d_{12}-PPP (8).

Synthetic mixture of TG from FA. FA (all >99% pure) were purchased from Nu-Chek-Prep. Five FA were used: P, S, O, L, and Ln. The mixed FA TG standard was prepared by the *p*-toluenesulfonic acid-catalyzed esterification of glycerol with an equimolar mixture of the FA (8). Glycerol and *p*-toluenesulfonic acid (Fisher Scientific Co., Fair Lawn, NJ) were used as received. All solvents were HPLC quality and were used without further purification.

Natural samples. Refined, bleached, and deodorized SBO was obtained from PVO Foods, Inc. (St. Louis, MO) and was randomized using the sodium-catalyzed interesterification method of List *et al.* (9). Refined and deodorized native lard was obtained from Colfax (Pawtucket, RI.) The lard was randomized by Nabisco Foods (Indianapolis, IN) using a similar method for randomization (interesterification).

GC. FAME were analyzed using a Varian 3400 (Palo Alto, CA) gas chromatograph equipped with a Supelco (Belefonte Park, PA) SP2380 30 m × 0.25 mm i.d. capillary column. The conditions were: inlet temperature = 240°C; detector temperature = 280°C; initial temperature = 150°C; initial time = 35 min; ramp to 210°C at 3°C/min. Methyl esters were made by the sodium methoxide esterification according to the method of Glass (10).

Liquid chromatography (LC). Solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or EM Science (Gibbstown, NJ). Solvents were HPLC grade or the highest available quality and were used without further purification. The HPLC pump used for LC/APCI–MS was an LDC 4100 MS (Thermo Separation Products, Schaumburg, IL) quaternary pump system with membrane degasser. The columns used were an Adsorbosphere C18 (Alltech Associates, Deerfield, IL), 25 cm × 4.6 mm, 5 μm (12% carbon load) in series with an Adsorbosphere UHS C18 25 cm × 4.6 mm, 10 μm (30% carbon load). The flow rate throughout was 1 mL/min. A gradient solvent program with propionitrile (PrCN), dichloromethane (DCM), and acetonitrile (ACN) was used to separate the mixture of homogeneous TG containing d_{12}-tripalmitin as internal standard. The gradient used was as follows: initial—PrCN/DCM/ACN (45:20:35, by vol); linear from 15 to 20 min to PrCN/DCM/ACN (45:25:30, by vol), held until 35 min; linear from 35 to 40 min to PrCN/DCM/ACN (45:30:25, by vol), held until 95 min. Separations incorporating PrCN provided slightly better peak shapes than those without, but due to variations in lot-to-lot solvent quality, the change was made to a DCM/ACN system. APCI source temperature and gas flows were optimized to minimize the amount of undesirable adduct formation.

Separations of the synthetic randomized mixture of heterogeneous TG and of the randomized and normal SBO and lard samples were accomplished using gradient elution as follows: initial—ACN/DCM (65:35, vol/vol); linear from 20 to 25 min to ACN/DCM (60:40, vol/vol), held until 35 min; linear from 35 to 40 min to ACN/DCM (55:45, vol/vol), held until 50 min; linear from 50 to 60 min to ACN/DCM (55:45, vol/vol), held until 85 min 5 μL of each sample solution was injected. The column effluent was split so that ~850 mL/min went to an ELSD and ~150 μL/min went to the APCI interface. ELSD data were used only to confirm proper operation of the LC system; these data are not presented. The ELSD was an ELSD MKIII (Varex, Burtonsville, MD). The drift tube was set at 140°C, the gas flow was 2.0 standard liters per minute. High purity N_2 was used as the nebulizer gas.

The quantitative analysis by RP-HPLC with FID detection used a linear gradient of ACN/DCM 70:30 to 40:60 vol/vol over 120 min. Columns and conditions were as previously described (11). The gradient used for LC–FID could not be used for APCI–MS analysis because the mass spectrometer's LC software did not allow gradient runs over 99 min in length.

MS. A Finnigan MAT (San Jose, CA) SSQ 710C mass spectrometer fitted with an APCI source was used to acquire mass spectral data. The vaporizer was operated at 400°C, and

the capillary heater was operated at 265°C. The corona voltage was set at 6.0 μA throughout. Auxiliary and sheath gases were set to 35 psi and 5 mL/min, respectively. Spectra were obtained from *m/z* 400 to *m/z* 1000 or *m/z* 1100, with a scan time of 1.75 to 2.0 s. Preliminary data showed no substantial ions below *m/z* 400. Chromatograms were processed using three-point smoothing for graphical output, but no smoothing was applied during quantitation of extracted ion chromatograms. All mass spectra shown represent an average of spectra over the breadth of a chromatographic peak. Nominal masses, shown in mass spectra, were obtained by application of a mass defect of 0 mmu @ 0 amu to 700 mmu @ 1000 amu.

Lipase hydrolysis. Positional isomers were determined using lipase hydrolysis as previously described (12). Briefly, lipase (EC 3.1.1.3) was used to cleave the FA at glycerol 1,3 carbons, and the lipolysis products were separated using solid phase extraction. The FAME of the lipolysis products were analyzed using GC–FID, and the composition obtained was compared to the FAME composition obtained for the intact samples.

Calculations. The expected composition of the synthetic mixture was calculated from the FA composition. If FA are esterified with glycerol, the number of possible TG molecular species, excluding isomers, can be calculated from the equation (13):

$$\text{number of TG molecular species} = \frac{n^3 + 3n^2 + 2n}{6} \quad [1]$$

where n is the number of FA. When 5 FA are used, 35 TG molecular species are possible. The 5 FA would be randomly distributed between all possible molecular species. Formulas for calculating the composition of a randomly distributed mixture of TG were derived from probability theory (14). The following equations were used to calculate the percentages of TG containing one (Eq. 2), two (Eq. 3), or three (Eq. 4) different FA:

$$\text{TAG\%} = \frac{\text{FA\%} \cdot \text{FA\%} \cdot \text{FA\%}}{10{,}000} \quad [2]$$

$$\text{TAG\%} = \frac{(\text{FA1\%} \cdot \text{FA1\%} \cdot \text{FA2\%}) \cdot 3}{10{,}000} \quad [3]$$

$$\text{TAG\%} = \frac{(\text{FA1\%} \cdot \text{FA2\%} \cdot \text{FA3\%}) \cdot 6}{10{,}000} \quad [4]$$

These equations also were used to calculate the theoretical compositions of the randomized SBO and lard samples from their FA compositions determined by calibrated GC–FID.

The percent relative errors for TG were calculated by subtracting the statistically expected amount from the determined amount, multiplying by 100, and then dividing by the statistically expected amount. The percent relative errors for the most abundant TG in a sample were averaged to give the average relative error. TG present at low levels had higher percent relative errors, so these were not included in calculation of the average relative error for a sample. Similarly, the percent relative errors for the FA was calculated by comparison of the FA composition calculated from the TG composition to the FA composition determined by calibrated GC–FID. The individual FA relative errors were averaged to give the average relative error for the FA composition.

RESULTS

Homogeneous mixture. Seven concentrations of a six-component homogeneous TG standard mixture were prepared, and raw quantitative data were obtained by summing the areas under the $[DG]^+$ and $[M + 1]^+$ peaks. The total areas under the $[DG]^+$ and $[M + 1]^+$ peaks were obtained from extracted ion chromatograms (EIC), in which a chromatogram was produced by selectively extracting the appropriate $[DG]^+$ and $[M + 1]^+$ *m/z* for each TG from the total, or reconstructed ion chromatogram.

Calibration curves were constructed by referencing the total peak areas for each TG to the area obtained for the deuterated d_{12}-PPP internal standard $[d_8\text{-DG}]^+$ peak having *m/z* = 559.5. Example calibration curves for three of the homogeneous TG standards are shown in Figure 1. The sensitivities (line slopes) and intercepts are given in slope/intercept form for the six components as follows: LnLnLn $y = 0.1201\,x + 0.05481$; LLL $y = 0.1199\,x + 0.05297$; OOO $y = 0.1471\,x + 0.05605$; SSS $y = 0.1337\,x + 0.06286$; PoPoPo $y = 0.2382\,x + 0.04958$; PPP $y = 0.1957\,x + 0.04113$, where y is the ratio of the area of the analyte peak to the area of the internal standard peak and x is the amount, in μg, actually injected. The correlation coefficients were LnLnLn =.9986, LLL = .9964, OOO = .9993, SSS = .9966, PoPoPo = .9987, PPP = .9998. As expected, the fit of the calibration curves was improved by decreasing the concentration ranges in the calibration curves. If only the lower four concentrations were used (0.01 μg/μL, 0.05 μg/μL, 0.10 μg/μL, and 0.50 μg/μL), the correlation coefficients became: LnLnLn = .9992; LLL = .9999; OOO = 1.0000; SSS = .9999; PoPoPo = 1.0000; PPP = 1.0000. PPP and PoPoPo had correlation coefficients of 1.0000 if the point obtained for 1.0 μg/μL was also included.

Of the 0.01 μg/μL solution, 5 μL contained 50 ng of each TG. This amount was the approximate detection limit of tristearin under these chromatographic conditions. The detection limit of tripalmitin was lower. Use of a micro flow chromatographic system with no splitting of the effluent stream would likely result in lower limits of detection.

35-Component synthetic TG mixture. An FID chromatogram and an annotated MS total ion chromatogram of the synthetic mixture of heterogeneous TG are shown in Figure 2. Although different gradients of ACN/DCM were used on the two systems, the chromatograms gave similar peak patterns. The shorter gradient run on the LC/APCI–MS system produced less resolution than the longer gradient used for the LC–FID run. The heated capillary interface between the atmospheric pressure and high vacuum regions of the ionization interface also resulted in a slight loss in peak resolution. In spite of higher resolution in the FID chromatogram, several peaks were present which contained unresolved overlapped TG. The TG which were unresolved in

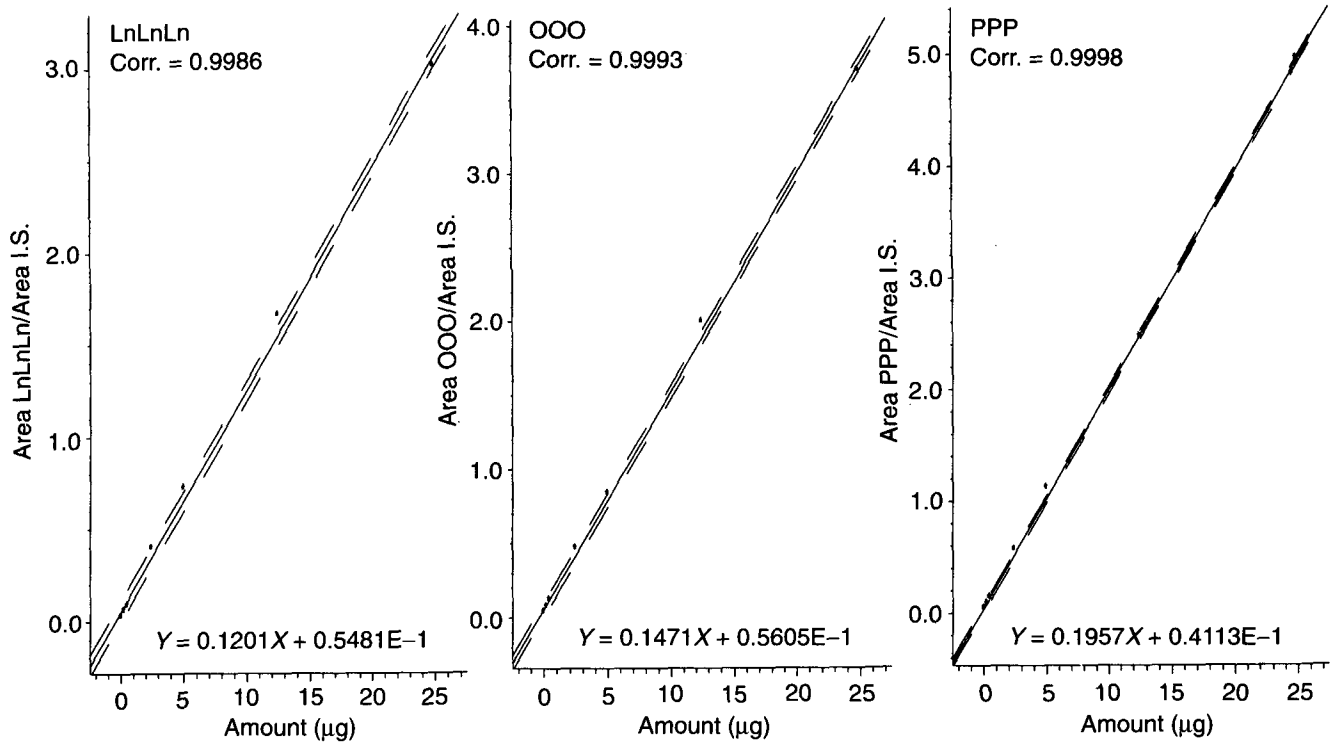

FIG. 1. Calibration curves for LnLnLn, OOO, and PPP where Ln is linolenic acid, O is oleic acid, and P is palmitic acid. Plots represent the total area for each triglyceride divided by the area of the internal standard peak, d_{12}-PPP, vs. the amount of each triglyceride injected. Line equations are given in slope/intercept form.

FID chromatograms were readily resolved by their different *m/z* in EIC. Because each TG produced up to three DG fragments, unresolved peaks in one EIC were resolved in other EIC of $[DG]^+$ arising from the same TG. The appearance of peaks in several EIC which all arose from one TG greatly facilitated the qualitative identification of individual TG in complex mixtures.

EIC of $[DG]^+$ *m/z* are shown in Figure 3. Each EIC showed an elution pattern which was based on the equivalent carbon number of the fatty acyl chains attached to the DG. The areas under the peaks in the $[DG]^+$ EIC were summed with the areas under peaks in EIC for $[M + 1]^+$ ions to provide a total area for each TG. The composition calculated from the combination of "raw" $[DG]^+$ and $[M + 1]^+$ ion areas for each TG in the EIC of the APCI–MS data is shown in Table 1. The composition determined by LC with FID detection is also given in Table 1. Positional isomers of the TG were not distinguished, so the abbreviations given in Table 1 identify molecular species without respect to the positional arrangement of the FA. Peaks in the EIC of *m/z* 603.5, arising from $[OO]^+$ and $[SL]^+$, were only partially resolved, so they were manually integrated to give the best approximation of appropriate peak areas. This partial overlap resulted in some variability, however, as demonstrated by the largest standard deviation in Table 1 shown by OLS. In the EIC of *m/z* 601.5, an unresolved overlap occurred between the "P" peak of $[OL]^+$ (#1) and the "O" peak of $[SLn]^+$ (#2). The ratios of the "Ln_1," "L_1," and "S_1" peaks to the "Ln_2," "L_2," and "S_2" peaks were used to apportion the overlapped peak, so that O_1/O_2 and P_1/P_2 were in the same proportion as $[(Ln_1 + L_1 + S_1)/(Ln_2 + L_2 + S_2)]$. Again, however, these individual peak areas represent only one of several peaks which contribute to the total area for a given TG molecular species.

The FA composition of the heterogeneous calibration mixture was determined (as the FAME) by GC with FID. The FAME mole percent composition was Ln = 18.5%; L = 19.8%; O = 20.2%; S = 20.6%; P = 20.9%. Using the above equations to calculate the randomly distributed TG percentage composition gave the statistical percentage composition shown in Table 1. The data in Table 1 demonstrate that the APCI–MS data require response factors to adjust the raw percentages to agree with the expected percentages. The areas under peaks attributable to the TG species (and the percentage composition) were dependent on the acyl chain length and on the fragmentation behavior of the TG, which was dependent on the number of sites of unsaturation in the TG species. Trisaturated TG produced virtually only $[DG]^+$ ion, with no $[M + 1]^+$ ion observed. Since the $[DG]^+$ ions, having lower *m/z*, were propagated through the MS system more efficiently than larger ions, they gave larger response than was observed for those TG which gave significant $[M + 1]^+$ ions. Conversely, TG containing polyunsaturated fatty acyl chains gave $[M + 1]^+$ ions as base peaks and showed less signal response. Other methods of mass spectrometric quantitation of TG have reported similar effects from unsaturation and/or chain length (15–19). Thus, Table 1 indicates that SSS was overrepresented, or gave a percentage composition larger than

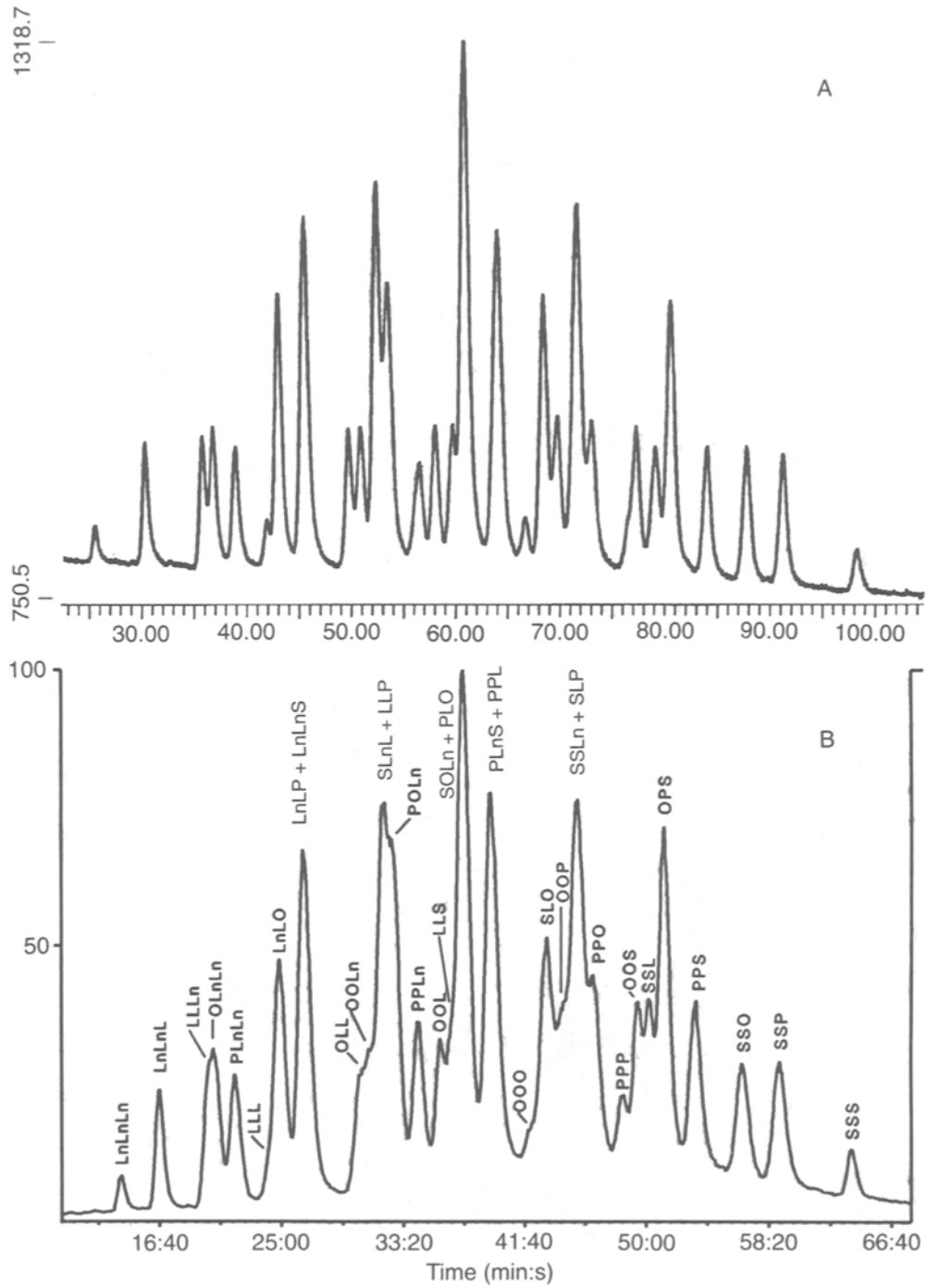

FIG. 2. A) Liquid chromatography–flame-ionization detection and B) liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatograms of a synthetic mixture of 35 triglycerides containing five randomly distributed fatty acids. Peak labels identify individual triglyceride molecular species. See Figure 1 for abbreviations; S, stearic acid; L, linoleic acid.

the calculated amount, while LnLnLn was underrepresented, or gave a percentage composition less than the calculated amount. PPP was overrepresented the most, as it had shorter acyl chains in addition to saturated acyl chains. Though not directly correlated, the same trend in dependence of the quantitative peak areas on the qualitative appearance of the spectra was seen in the sensitivities (slopes) of the calibration curves presented above. The sensitivity of the PPP calibration curve was larger than that for the SSS calibration curve, which was larger than that for the LnLnLn calibration curve. However, TG containing monounsaturated fatty acyl chains gave higher sensitivities than tri-saturates in the calibration curves.

Example mass spectra of four TG species are shown in

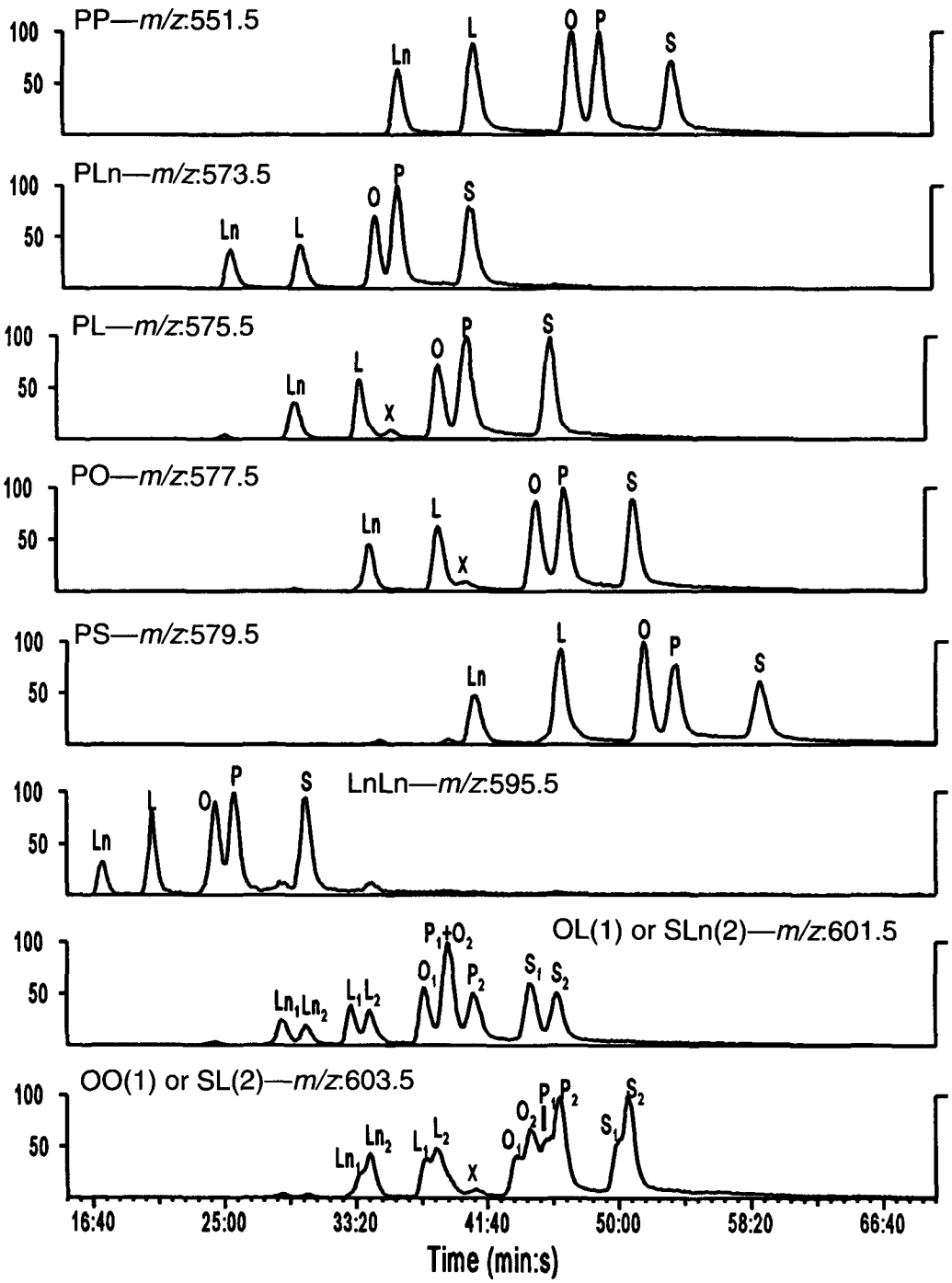

FIG. 3. Extracted ion chromatograms of [diglyceride (DG)]$^+$ *m/z* from a synthetic mixture of 35 triglycerides. [DG]$^+$ identity and *m/z* are given in the upper left of each chromatogram. The peaks are labeled with the identity of the fatty acid which, in combination with the [DG]$^+$, make each of the triglyceride molecular species. See Figures 1 and 2 for other abbreviations.

Figure 4. These spectra demonstrate that, as previously reported, spectra of TG containing more than four sites of unsaturation had [M + 1]$^+$ ions as base peaks, while spectra of TG containing less than three sites of unsaturation had [DG]$^+$ ions as base peaks. Though conditions were optimized to minimize the amount of PrCN adducts formed, these spectra showed distinct PrCN adduct ions. The separations carried out using only ACN/DCM gradients resulted in spectra containing virtually no adduct ions.

One method to produce response factors was to calculate a set of empirical factors for each TG which, when multiplied by the raw MS composition, gave the statistical composition.

TABLE 1
Percentage Composition of Heterogeneous TG Synthetic Mixture

TG[a]	Average raw MS % (n = 3)[b]	Statistical % from GC–FID[c]	GC–FID RF adjusted %[d]	Average LC–FID % (n = 3)
POS	6.3 ± 0.5	5.2	5.5	5.7 ± 0.2
PLS	5.8 ± 0.2	5.1	5.0	6.3 ± 0.2
PLO	4.5 ± 0.2	5.0	4.4	6.9 ± 0.3
LOS	4.4 ± 0.7	5.0	5.0	4.9 ± 0.2
PLnS	4.7 ± 0.3	4.8	4.7	4.1 ± 0.1
PLnO	4.5 ± 0.3	4.7	4.3	5.5 ± 0.2
LnOS	4.1 ± 0.3	4.6	4.3	4.3 ± 0.2
PLnL	4.5 ± 0.2	4.6	4.8	5.5 ± 0.2
LnLS	3.7 ± 0.1	4.5	4.1	3.8 ± 0.1
LnLO	3.8 ± 0.4	4.4	4.9	4.8 ± 0.3
PPS	3.6 ± 0.5	2.7	2.8	2.3 ± 0.2
SSP	3.0 ± 0.3	2.7	2.5	2.3 ± 0.3
PPO	3.7 ± 0.3	2.6	2.7	2.9 ± 0.1
PPL	3.1 ± 0.2	2.6	2.5	3.3 ± 0.1
SSO	2.9 ± 0.3	2.6	2.6	2.6 ± 0.1
OOP	2.9 ± 0.3	2.6	2.5	2.7 ± 0.0
SSL	2.8 ± 0.2	2.5	2.8	2.3 ± 0.1
OOS	2.5 ± 0.0	2.5	2.7	2.6 ± 0.1
LLP	2.4 ± 0.1	2.5	2.3	3.1 ± 0.1
LLS	2.2 ± 0.0	2.4	2.3	1.7 ± 0.1
OOL	2.5 ± 0.1	2.4	2.7	2.0 ± 0.2
PPLn	3.1 ± 0.2	2.4	2.5	1.8 ± 0.1
LLO	2.3 ± 0.1	2.4	2.8	1.9 ± 0.0
SSLn	2.3 ± 0.2	2.4	2.4	1.9 ± 0.1
OOLn	1.7 ± 0.1	2.3	2.0	2.1 ± 0.1
LLLn	1.9 ± 0.2	2.2	2.5	1.8 ± 0.1
LnLnP	1.9 ± 0.1	2.1	2.2	2.1 ± 0.1
LnLnS	1.8 ± 0.1	2.1	2.4	1.7 ± 0.1
LnLnO	1.5 ± 0.0	2.1	1.9	2.4 ± 0.1
LnLnL	1.4 ± 0.2	2.0	2.0	1.7 ± 0.2
PPP	1.5 ± 0.1	0.9	1.0	0.5 ± 0.1
SSS	1.0 ± 0.2	0.9	0.8	0.8 ± 0.1
OOO	0.7 ± 0.2	0.8	0.8	0.5 ± 0.0
LLL	0.5 ± 0.1	0.8	0.6	0.5 ± 0.1
LnLnLn	0.4 ± 0.0	0.6	0.6	0.5 ± 0.0
Sum	100.0	100.0	100.0	100.0
Avg. relative error %	17.0	—	7.4	17.4

[a]Abbreviations: TG, triglyceride; P, palmitic acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; RF, response factor. TG names do not indicate positional location of fatty acids (FA) in TG.
[b]TG composition obtained from the sum of areas under diglyceride and $[TG + 1]^+$ peaks in extracted ion chromatograms from liquid chromatography (LC)/atmospheric pressure chemical ionization–mass spectrometry (MS) data.
[c]TG composition calculated from the FA composition determined by gas chromatography–flame-ionization dection (GC–FID), assuming a random distribution of FA.
[d]TG composition obtained by multiplying raw composition by response factor calculated in two steps: (i) response factor for each FA was calculated by dividing the FA percentage determined by GC–FID by the FA percentage calculated from the raw TG composition determined by LC/MS; (ii) response factors for each TG were obtained by multiplying together FA response factors for each FA in the TG.

TABLE 2
Fatty Acid Percentage Composition of Heterogeneous TG Synthetic Mixture[a]

FA	From raw MS TG[b]	From calibrated GC–FID	From GC–FID-adjusted TG[c]	From LC–FID TG[d]
P	24.0	20.9	20.9	22.1
Ln	16.3	18.5	18.2	17.6
L	18.5	19.8	19.2	20.0
O	19.8	20.2	20.7	20.8
S	21.4	20.6	20.9	19.4
Sum	100.0	100.0	100.0	100.0
Avg. relative error %	8.0	—	1.7	4.0

[a]Abbreviations as in Table 1.
[b]FA composition calculated from the TG composition determined by LC/MS from the sum of $[DG]^+$ and $[TG + 1]^+$ peaks.
[c]FA composition calculated from the adjusted TG composition which was obtained by application of response factors derived from the ratio of FA compositions of the raw MS data and GC–FID data.
[d]FA composition calculated from the TG composition determined using LC with FID detection.

This set of response factors for each TG could then be applied to other TG mixtures containing a similar set of TG. Response factors obtained from the synthetic mixture were applied to the randomized SO sample presented below.

A second method for producing response factors was to compare the FA composition calculated from the TG composition to the FA composition determined by calibrated GC–FID. The FA compositions determined by GC–FID and calculated from the "raw" MS data are given in Table 2. The amount of palmitic acid calculated from the TG composition was larger than the amount determined by GC–FID, as was the amount of stearic acid. The amount of linolenic acid calculated from the TG composition was less than that determined by GC–FID. Response factors for each FA were calculated by dividing the FA composition determined by calibrated GC with FID detection by the FA composition calculated from the TG composition given from the "raw" APCI–MS data. These numbers were then normalized to the smallest value to produce a set of factors: P = 1.00; S = 1.12; O = 1.20; L = 1.19; Ln = 1.29. Response factors for each TG were calculated by simply multiplying the appropriate FA response factors together. For example, the response factor for PPP was $1.00 \cdot 1.00 \cdot 1.00 = 1.00$, while that for LLL was $1.19 \cdot 1.19 \cdot 1.19 = 1.68$. Application of the response factors calculated from the GC–FID FA composition gave the TG composition listed in Table 1.

It can be seen that the TG composition adjusted using the GC–FID-derived response factors agrees more closely with the calculated composition than does the composition determined by LC–FID, which is accepted for use for quantitation without response factors (20). The average relative error for the GC–FID-adjusted APCI–MS TG composition was 7.4% per TG compared to the predicted composition. The average relative error of the TG composition determined by LC–FID was 17.4% per TG compared to the predicted composition. The composition determined by LC–FID had an average relative error very similar in magnitude to the "raw" APCI–MS composition. The FA composition resultant from the response-factor-adjusted APCI–MS TG composition, Table 2, had an average relative error of 1.7% per FA compared to the FA composition determined by GC–FID. The FA composi-

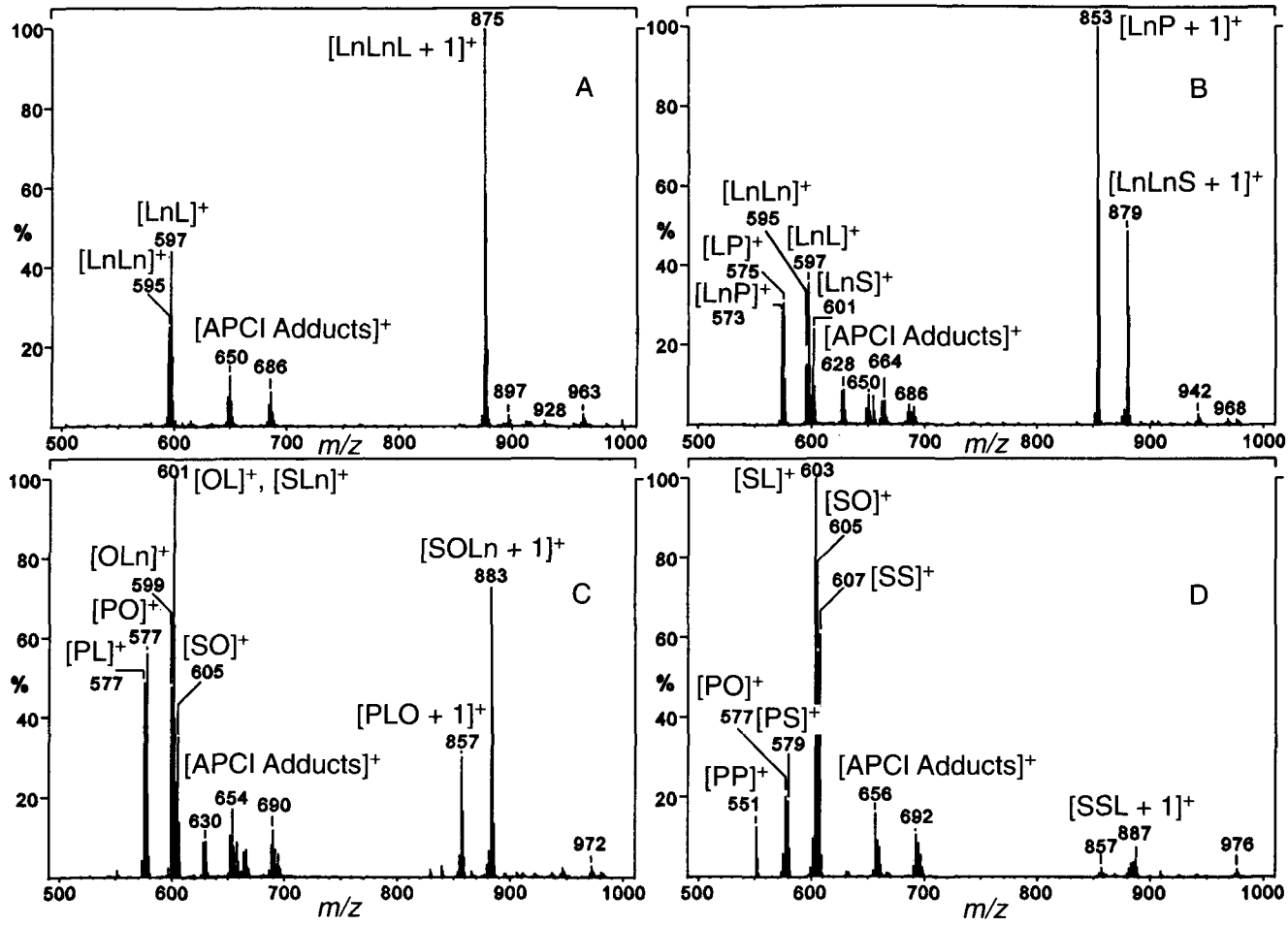

FIG. 4. Mass spectra of A) LnLnL (8 sites), B) LnLP and LnLnS (5 and 6 sites), C) PLO and SOLn (3 and 4 sites), and D) SSL with smaller amounts of OOS and OPS (2, 1 sites). Adducts formed were the $[DG + 53]^+$ and $[DG + 89]^+$ ions derived from addition of propionitrile [molecular weight (MW) = 55 amu)] and from addition of propionitrile plus acetonitrile (MW = 38 amu). See Figures 1, 2, and 3 for abbreviations; APC1, atmospheric pressure–chemical ionization.

tion resultant from the TG composition obtained from the LC–FID data had an average relative error of 4.0% per FA.

Randomized SBO. Chromatograms of randomized SBO obtained using LC–FID and LC/APCI–MS are shown in Figure 5. These two methods gave very similar peak patterns and comparable visible resolution. EIC were used to quantitate the $[DG]^+$ and $[M + 1]^+$ peak areas as described above. Example EIC are shown in Figure 6. Example mass spectra are shown in Figure 7. Because the chromatographic separation used an ACN/DCM gradient, these spectra did not show significant adduct formation. Only small amounts of ACN adducts were formed. The "raw" TG composition calculated from the sum of $[DG]^+$ and $[M + 1]^+$ peaks is given in Table 3, as is the composition determined by LC–FID. The theoretical TG composition of the randomized SBO was calculated using the FA composition determined by calibrated GC–FID, given in Table 4. The calculated TG composition is given in Table 3. The FA composition at carbon 2 of the TG determined by GC–FID of the products of lipase hydrolysis is given in Table 4, and may be compared to the composition at carbon 2 of normal SBO. The change in composition at carbon 2 toward that of the total FA composition indicated that randomization was mostly, but not absolutely, complete.

Response factors were produced by comparison of the FA composition calculated from the "raw" LC/APCI–MS data to the FA composition determined by GC–FID. The normalized response factors for the FA were as follows: P = 1.13; S = 1.00; O = 1.36; L = 1.87; Ln = 1.07. As above, these FA response factors were multiplied together to give TG response factors for each of the TG molecular species. The adjusted TG composition obtained using the FA-derived response factors is given in Table 3. These response factors generally reflected the qualitative fragmentation behavior discussed above except for the response factor for linolenic acid. Based on the considerations mentioned above, it was expected that this FA would be underrepresented and require a response factor greater than those for oleic and linoleic acids. In this SBO sample, in contrast to the synthetic mixture, linoleic acid

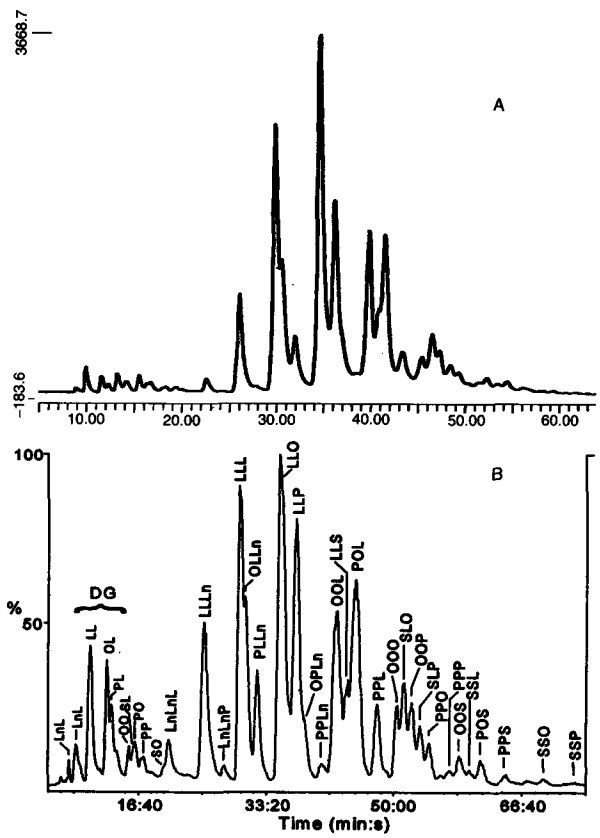

FIG. 5. A) Liquid chromatography–flame-ionization detection and B) liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatograms of randomized soybean oil. Peak labels identify individual DG and triglyceride molecular species. DG, formed during the randomization, eluted first. See Figures 1, 2, and 3 for abbreviations.

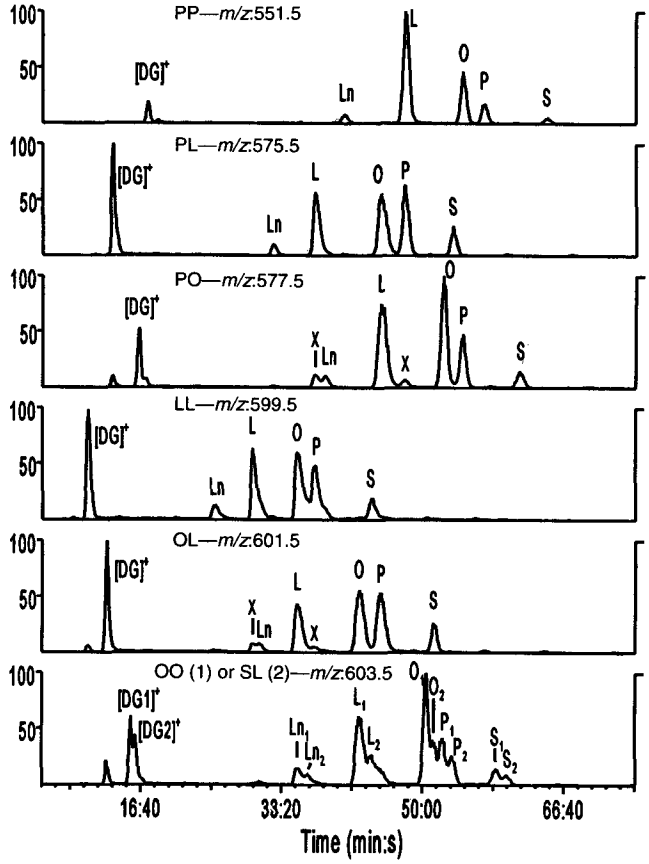

FIG. 6. Extracted ion chromatograms of $[DG]^+$ *m/z* from randomized soybean oil. $[DG]^+$ identity and *m/z* are given in the upper left of each chromatogram. The peaks are labeled with the identity of the fatty acid which, in combination with the $[DG]^+$, make each of the triglyceride molecular species. See Figures 1, 2, and 3 for abbreviations.

was present in a much larger percentage than was linolenic acid. Because of the elution patterns in the EIC, the "L" peak in one EIC gave a ^{13}C isotope peak, for molecules containing two ^{13}C atoms, at nearly the same chromatographic position as the "Ln" peak in the EIC for a DG having one less site of unsaturation (for instance, the "L" peak of the $[PL]^+$ EIC at *m/z* 575.5 gave an isotope peak at the same location as the "Ln" peak in the *m/z* 577.5 EIC of $[PO]^+$). The molecules which contained two ^{13}C isotopes therefore contributed peak area to the peaks in the EIC of the DG having one less site of unsaturation. When linoleic acid was present in a much larger amount than linolenic acid, the contribution of peak area from the isotope-containing molecules caused overestimation of the amount of linolenic acid-containing species. Imperfect chromatographic overlap of the peaks and some small variability in isotope ratios made subtraction of a calculated isotope area impractical. The response factors calculated from the FA composition or from the statistical composition effectively compensated for this overestimation.

The adjusted TG composition obtained using FA-derived response factors was in fair agreement with the composition calculated for a random distribution of the FA. The average relative error for the twenty most abundant molecular species was 8.6% per TG for the TG composition obtained from the GC-adjusted APCI–MS data, compared to the composition expected from a random distribution. The average relative error for the most abundant TG molecular species was 23.9% for the TG composition obtained by LC with FID detection, compared to the TG composition expected from a random distribution. TG molecular species which were not detected using LC–FID, which had relative errors of 100%, were not included in the calculation of the average percent relative error. The FA compositions calculated from the TG compositions obtained from the GC-adjusted MS data and the LC–FID data are given in Table 4. The FA composition calculated from the TG composition obtained from the GC-adjusted MS data had an average relative error of 2.2% per FA compared to the FA composition obtained by GC–FID. The FA composition calculated from the TG composition obtained by LC–FID had an average relative error of 6.5% per FA.

Quantitation based on response factors calculated from the synthetic mixture was also performed. Relative response factors for each TG were calculated by dividing the percentage expected from the random distribution of FA by the TG per-

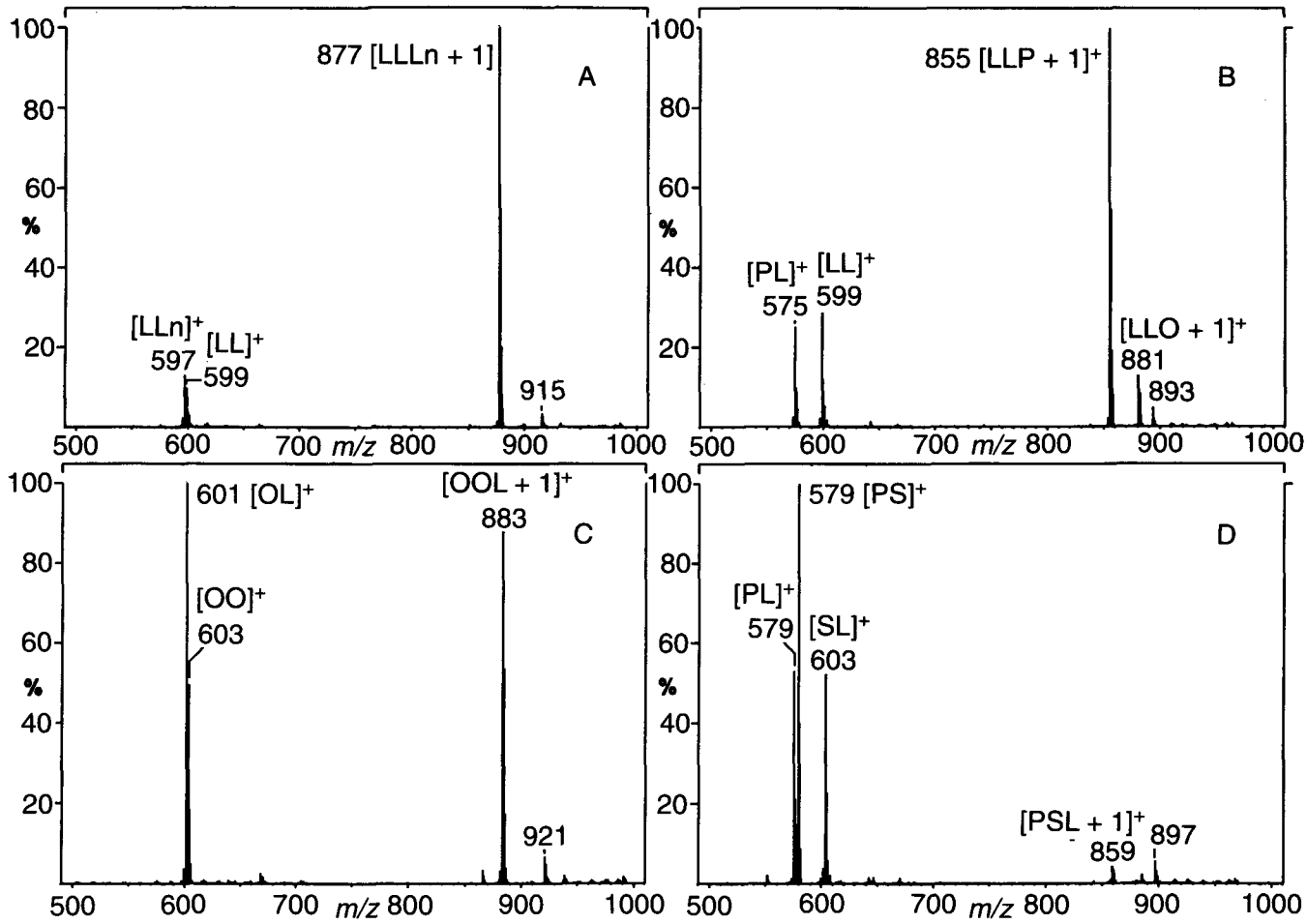

FIG. 7. Mass spectra of A) LLLn (7 sites), B) LLP with a small amount of LLO (4 sites), C) OOL (4 sites), and D) PSL (2 sites). Small amounts of [DG + 38]$^+$ adduct ions derived from addition of acetonitrile (MW = 38 amu) were formed. See Figures 1, 2, and 3 for abbreviations.

centage obtained by APCI–MS. Application of response factors thus obtained to the randomized SBO resulted in the TG composition given in Table 3. The poor agreement of the calculated composition with the expected composition indicated that relative response factors calculated from a sample with a significantly different percentage composition were not useful. The concentration dependence of the relative response factors necessitated use of response factors derived from mixtures of similar composition. The FA composition calculated from the TG composition obtained using response factors obtained from the synthetic mixture had an average relative of 26.7% per FA. Because of the demonstrated lack of utility, the use of response factors calculated from the synthetic mixture was not pursued further.

Normal SBO. A chromatogram obtained using LC with APCI–MS detection is shown in Figure 8. The TG composition obtained from this chromatogram is given in Table 5, listed as "raw" APCI–MS data. The TG composition determined by LC–FID is also given in Table 5. Quantitation using response factors derived from the FA composition determined by GC–FID was performed as described above. The FA composition calculated from the "raw" MS data and the FA composition determined by GC–FID are given in Table 6. The response factors calculated by comparison of the MS to the GC–FID data were: P = 1.12; S = 1.12; O = 1.44; L = 2.03; Ln = 1.00. Response factors for individual TG molecular species were calculated as described above. The TG composition which resulted from application of the response factors thus derived is given in Table 5, referred to as "GC–FID RF Adjusted %."

The FA composition obtained from the TG composition calculated using response factors derived from GC–FID data is given in Table 6. This FA composition showed an average relative error of 4.5% per FA, compared to 19.8% average relative error from the "raw" APCI–MS data and 9.2% average relative error per FA from the LC–FID data.

Quantitation was also performed using response factors calculated from the randomized SBO. Response factors for each of the TG molecular species were calculated by dividing the statistically expected composition for the randomized

TABLE 3
Percentage Composition of Randomized Soybean Oil TG[a]

TG	Raw MS %	Statistical % from GC–FID	GC–FID RF adjusted %	LC–FID %	Mixture adjusted %[b]
LLO	13.8	20.5	19.9	20.5	12.8
LLL	6.6	14.7	13.2	12.9	9.1
OOL	8.3	9.6	8.8	8.4	7.6
LLP	8.7	9.7	10.4	11.4	8.3
PLO	10.6	9.1	9.3	11.1	11.1
LLLn	6.0	5.9	6.8	5.9	6.2
LnLO	7.0	5.5	5.8	7.6	7.2
LLS	3.8	3.3	4.0	2.0	3.8
LOS	3.7	3.0	2.9	3.4	4.2
PLnL	3.4	2.6	2.3	3.7	3.1
OOP	3.4	2.1	2.2	1.8	3.0
PPL	3.4	2.1	2.4	2.0	2.5
OOO	2.4	1.5	1.8	1.4	3.4
PLS	2.4	1.4	1.5	1.0	1.9
OOLn	2.1	1.3	1.3	0.8	2.6
PLnO	2.6	1.2	1.3	1.6	2.6
LnLS	1.3	0.9	0.8	0.0	1.5
PPO	1.8	1.0	0.9	0.7	1.2
LnLnL	1.2	0.8	0.8	0.7	1.4
OOS	1.4	0.7	0.8	0.8	1.3
POS	1.2	0.7	0.6	0.6	0.9
LnOS	0.9	0.4	0.4	0.0	0.9
LnLnO	0.8	0.4	0.4	0.3	1.0
SSL	0.5	0.2	0.3	0.4	0.4
PPLn	0.5	0.3	0.2	0.0	0.4
PLnS	0.4	0.2	0.1	0.0	0.3
LnLnP	0.3	0.2	0.1	0.0	0.3
PPS	0.3	0.2	0.1	0.3	0.2
PPP	0.3	0.2	0.1	0.1	0.2
SSO	0.3	0.1	0.1	0.1	0.2
LnLnS	0.1	0.1	0.0	0.0	0.1
SSP	0.1	0.1	0.0	0.1	0.1
LnLnLn	0.1	<0.05	<0.05	0.2	0.1
SSLn	0.1	<0.05	<0.05	0.0	0.1
SSS	<0.05	<0.05	<0.05	0.1	0.0
Other	0.4	0.3	0.2	0.10	0.0
Sum	100.0	100.0	100.0	100.0	100.0
Av. rel. err. % ($n = 20$)[c]	46.4	—	8.6	23.9	46.5

[a]Abbreviations and column headings as in Table 1.
[b]TG composition obtained by application of response factors calculated from the synthetic mixture. Response factors were calculated by dividing the TG amount calculated from a random distribution of the FA in the synthetic mixture by the TG amount determined by LC/MS.
[c]Average relative error for the twenty most abundant TG species.

TABLE 4
Fatty Acid Percentage Composition of Randomized Soybean Oil TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	TG position 2[b]
Po	0.2	0.1	0.1	0.0	0.0
P	15.4	11.6	11.9	12.5	13.8
Ln	9.8	7.0	7.3	7.4	4.5
L	42.0	52.7	52.2	52.3	49.5
O	26.8	24.6	24.5	24.6	27.7
S	5.8	3.9	4.1	3.2	4.5
Sum	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	29.9	—	2.2	6.5	—

[a]Abbreviations as in Table 1. Column headings as in Table 2.
[b]FA composition at glycerol carbon 2 determined by GC–FID analysis of the products of lipase hydrolysis. Perfect agreement with the total FA composition determined by GC–FID indicates complete randomization.

sample by the raw APCI–MS percentage composition. The response factors thus obtained were applied to the raw APCI–MS data for the normal SBO. The resultant TG composition is given in Table 5. The TG composition agrees very well with the TG composition obtained using response factors calculated from the GC–FID composition determined for the normal SBO. The FA composition calculated from the randomized-SBO-normalized TG composition, shown in Table 6, exhibited an average relative error of 6.5% per FA. This compares favorably with the results obtained from the GC–FID-derived response factor normalized data.

Despite the close similarity in the results obtained using response factors derived from randomized SBO vs. GC–FID data, the methods used to obtain the response factors were very different. The randomized SBO-derived response factors were dependent only on the GC–FID composition and statistical distribution determined for the randomized SBO sample, with no dependence on the GC composition actually determined for the normal SBO. On the other hand, the GC–FID-derived response factors had no dependence on the randomized SBO sample or on any statistical calculation, but depended only on the GC–FID data for the normal SBO. That the two approaches resulted in very similar TG compositions, and yielded similar calculated FA compositions indicated that both quantitation methods may be used to produce results which provide less average relative error than LC–FID results.

Randomized lard. An LC/APCI–MS chromatogram of randomized lard is shown in Figure 9. The TG composition determined from this chromatogram is given in Table 7, as is the composition determined by LC–FID. Because this was a ran-

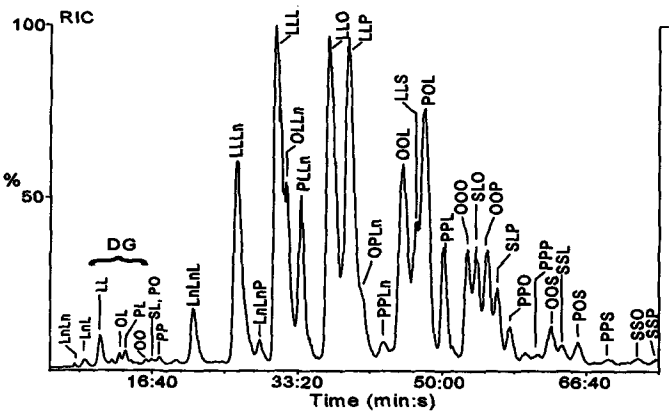

FIG. 8. Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatogram of normal soybean oil. Peak labels identify DG or triglyceride molecular species. RIC, reconstructed ion chromatogram. See Figures 1, 2, and 3 for abbreviations.

TABLE 5
Percentage Composition of Normal Soybean Oil (SBO) TG[a]

TG	Raw MS %	GC–FID RF adjusted %	Randomized-SBO-adjusted %[b]	LC–FID %
LLO	11.2	17.7	17.3	16.4
LLL	6.1	13.6	14.0	15.3
LLP	9.7	11.8	11.2	14.0
OOL	7.6	8.5	9.0	8.3
PLO	9.2	7.9	8.1	10.1
LnLO	8.2	6.3	6.6	6.1
LLLn	6.1	6.7	6.2	6.5
LLS	4.0	4.9	3.6	3.3
PLnL	4.1	2.5	3.3	3.9
LOS	3.4	2.9	2.9	3.1
OOP	4.0	2.5	2.5	0.9
PPL	3.5	2.4	2.3	2.2
OOO	3.2	2.5	2.1	3.0
PLnO	3.5	1.5	1.7	0.9
PLS	2.3	1.6	1.5	1.2
OOLn	2.1	1.2	1.3	0.8
LnLnL	1.5	0.8	1.0	0.9
LnLS	1.4	0.8	0.9	0.0
OOS	1.6	1.0	0.8	0.8
PPO	1.3	0.6	0.8	0.5
POS	1.0	0.5	0.6	0.2
LnLnO	1.1	0.4	0.5	0.4
LnOS	1.0	0.4	0.5	0.0
SSL	0.5	0.4	0.3	0.3
PPLn	0.5	0.1	0.3	0.0
LnLnP	0.4	0.1	0.2	0.0
PLnS	0.4	0.1	0.2	0.0
LnLnS	0.2	0.1	0.1	0.0
SSO	0.3	0.1	0.1	0.0
LnLnLn	0.1	<0.05	0.1	0.1
SSLn	0.1	<0.05	<0.05	0.0
PoPL	0.1	<0.05	<0.05	0.0
PoPO	<0.05	<0.05	<0.05	0.0
PPP	<0.05	<0.05	<0.05	0.2
PPS	<0.05	<0.05	<0.05	0.1
Other	0.3	<0.05	<0.05	0.3
Sum	100.0	100.0	100.0	100.0

[a]Abbreviations and column headings as in Table 1.
[b]TG composition obtained by application of response factors calculated from randomized soybean oil. Response factors were calculated by dividing the expected TG composition (calculated based on random distribution of FA) by the TG composition determined by LC/MS.

TABLE 6
Fatty Acid Percentage Composition of Normal Soybean Oil (SBO) TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	From random.-SBO-adjusted TG[b]	TG position 2
Po	0.2	0.0	0.0	0.0	0.0	0.0
P	15.2	10.9	11.6	12.6	12.0	1.2
Ln	11.4	7.3	7.5	7.1	8.3	6.4
L	40.7	53.0	52.3	54.2	51.5	66.3
O	26.8	24.8	24.1	22.8	24.2	24.6
S	5.7	4.1	4.4	3.4	4.0	1.4
Sum	100.0	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	19.8	—	4.5	9.2	6.5	—

[a]Abbreviations as in Table 1, column headings as in Table 4.
[b]FA composition calculated from the TG composition adjusted using randomized soybean oil-derived response factors.

domized sample, the theoretical composition could be calculated from the statistical distribution of FA. The FA composition determined by calibrated GC–FID used for calculation of the statistically expected TG composition is given in Table 8. The expected TG composition calculated from the FA composition is given in Table 7. Comparison of the FA composition at carbon 2 of the TG of the randomized lard to the total FA composition, Table 8, indicated that the lard was well randomized, in contrast to the FA composition at carbon 2 for the normal lard. The FA composition calculated from the "raw" TG composition determined by APCI–MS is given in Table 8. The FA composition determined by calibrated GC–FID was divided by the FA composition calculated from the "raw" TG composition to produce the following response factors for each of the FA: M = 1.66; Po = 1.00; P = 1.80; L = 1.90; O = 2.01; S = 1.73. Response factors for each of the TG molecular species was obtained by multiplying the appropriate FA response factors together, as described above. The TG composition resultant from application of the TG response factors derived from these FA response factors is given in Table 7. Because of the small amounts of linolenic acid and C_{20} fatty acids distributed throughout possible TG molecular species, these were not quantitated in the EIC for the lard samples, but several molecular species were identified by their fragments and pseudo-molecular ions. The average relative error for the 35 components which made up 99.6% of the quantitated composition was 10.1% per TG for the GC–FID-adjusted composition, compared to 31.4% per TG and 28.9% per TG for compositions determined by unadjusted APCI–MS data and LC–FID data, respectively. TG species which were not identified by LC–FID, and so had 100% relative error, were not included in the calculation of the average relative error for that detection method. Numerous species present at near 1% in composition were not identified in the LC–FID chromatograms because they eluted undetected under larger peaks. Many such species were conclusively identified from APCI–MS data, using EIC.

The FA compositions which were calculated from the TG compositions reported in Table 7 are shown in Table 8. As was the case for all samples discussed above, the average relative error for the FA composition calculated from the GC–FID-adjusted TG composition showed the least average relative error of the quantitation methods. The FA composition calculated from the LC–FID TG composition exhibited lower amounts of myristic and palmitoleic acids present than were determined by calibrated GC–FID. This is a result of the inability of the LC–FID detector to resolve the many minor components containing these FA which coeluted with components present in larger amounts. The EIC obtained from the APCI–MS data allowed ready identification, by $[DG]^+$ and $[M + 1]^+$ *m/z*, of numerous myristic and palmitoleic acid-containing species which coeluted with major species. This re-

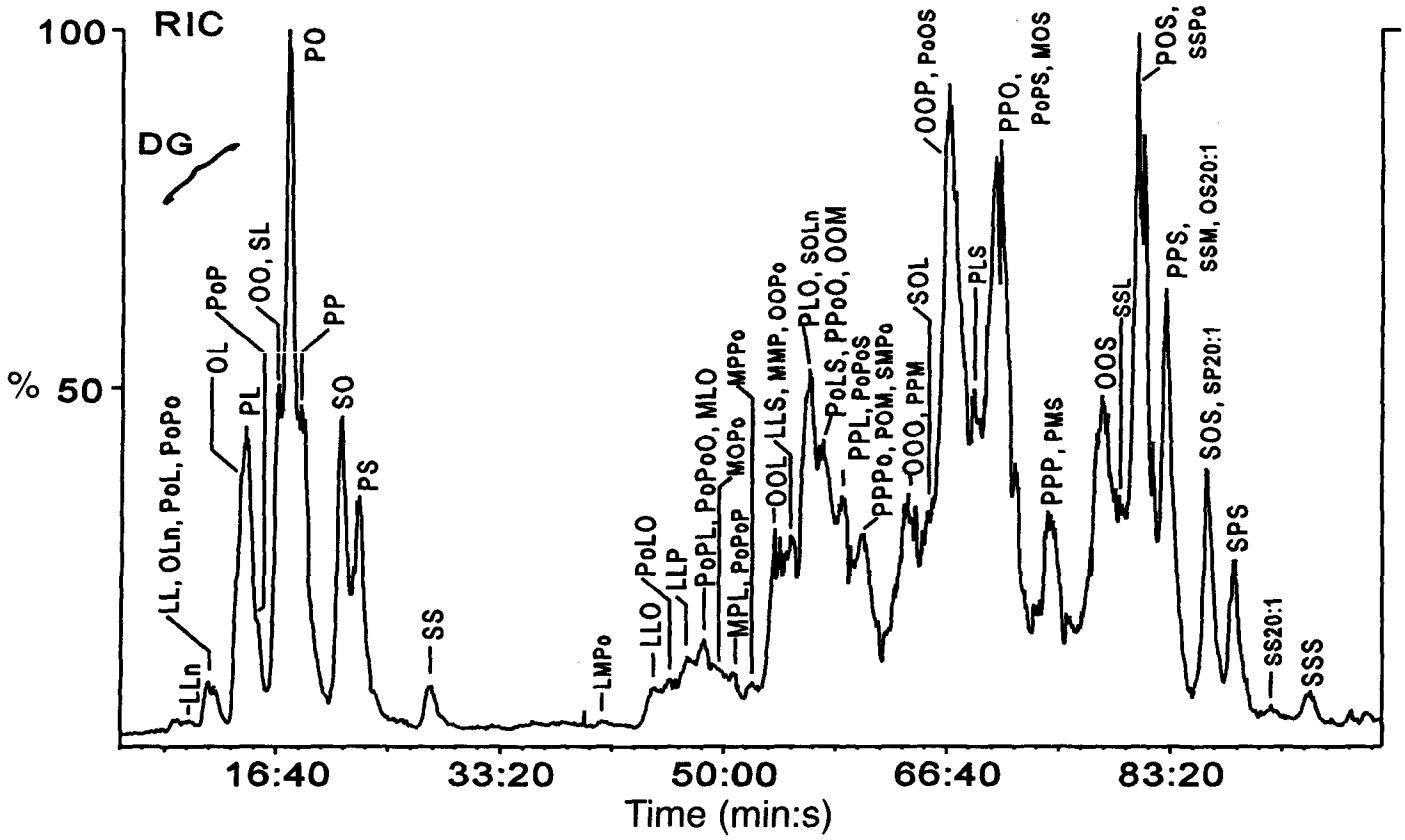

FIG. 9. Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatogram of randomized lard. Peak labels identify individual DG and triglyceride molecular species. DG, formed during the randomization, eluted first. See Figures 1, 2, 3, and 8 for abbreviations.

sulted in excellent agreement between the response factor-adjusted APCI–MS data and the GC–FID data.

Normal lard. A chromatogram obtained by LC/APCI–MS of normal lard is shown in Figure 10. The TG composition determined from this chromatograms is given in Table 9. As in the case of normal SBO, two methods for calculation of response factors were used for quantitation of normal lard. Response factors were calculated by comparison of the FA composition determined by GC–FID, given in Table 10, to the FA composition calculated from the TG composition, and response factors were calculated from comparison of the theoretical composition of randomized lard to the composition determined by APCI–MS. The FA response factors calculated by dividing the FA composition calculated from the TG composition determined by APCI–MS by the FA composition determined by GC–FID, normalized to the smallest, were: M = 1.79; Po = 1.00; P = 1.84; L = 1.92; O = 1.91; S = 1.50. Response factors for each of the TG molecular species were obtained by multiplication of the appropriate FA response factors. The resultant response factor-adjusted TG compositions are given in Table 9. The adjusted APCI–MS compositions are in good agreement with each other, in contrast to the composition obtained using LC–FID. As with the randomized lard, numerous TG species present in amounts as high as 1.5% were not identified by LC–FID because they eluted undetected under larger chromatographic peaks. As with the randomized lard, this resulted in a FA composition calculated from the LC–FID TG composition which contained smaller amounts of myristic and palmitoleic acids than determined by GC–FID. The FA compositions calculation from the response-factor-adjusted APCI–MS data showed good agreement with the FA composition determined by calibrated GC–FID.

DISCUSSION

The analysis of the mono-acid TG presented above demonstrated that, for individual TG of interest, calibration curves having good correlation coefficients could be constructed. Even simple natural fats and oils, however, contain enough TG molecular species that it becomes impractical to construct calibration curves for all species of interest. Thus, alternative quantitation approaches were necessary which were targeted at more complicated samples. The trends observed in the slopes of the calibration curves, in correlation with the trends observed in the MS fragmentation patterns of TG with varying amounts of unsaturation, demonstrated that the quantitation of TG is distinctly related to the qualitative appearance of the spectra obtained using APCI–MS.

Quantitation of a complex sample generally depends on

TABLE 7
Percentage Composition of Randomized Lard TG[a]

TG	Raw MS % (n = 6)	Statistical % from GC–FID	GC–FID RF adjusted %	LC–FID %
OOP	12.3 ± 0.9	14.8	14.6	17.5
POS	11.2 ± 0.8	12.1	11.4	12.4
PPO	10.3 ± 0.9	10.9	10.9	12.5
OOS	7.4 ± 0.3	8.3	8.4	12.4
OOO	5.6 ± 1.2	6.7	7.3	7.5
PLO	4.7 ± 0.4	5.7	5.3	5.8
PPS	5.3 ± 0.7	4.5	4.8	3.9
OOL	3.7 ± 0.4	3.9	4.6	3.4
SSO	3.9 ± 0.4	3.4	3.8	2.9
LOS	2.5 ± 0.3	3.2	2.7	4.0
PPP	3.2 ± 0.3	2.7	3.0	1.4
SSP	2.8 ± 0.4	2.5	2.5	1.6
PLS	2.3 ± 0.3	2.4	2.2	3.4
PPL	2.0 ± 0.3	2.1	2.0	1.0
PoPO	3.2 ± 0.4	2.0	1.9	2.9
MPO	1.5 ± 0.3	1.4	1.5	0.0
OOPo	1.8 ± 0.2	1.3	1.2	0.0
PoOS	1.9 ± 0.3	1.1	1.1	0.0
OOM	0.8 ± 0.1	1.0	0.8	0.0
PoPS	1.8 ± 0.1	0.8	0.9	0.0
MOS	0.7 ± 0.1	0.8	0.7	0.0
LLO	1.0 ± 0.2	0.8	1.1	0.5
PPPo	1.4 ± 0.3	0.7	0.7	0.0
SSL	0.7 ± 0.1	0.7	0.7	0.0
MPS	0.7 ± 0.1	0.6	0.6	0.0
LLP	0.5 ± 0.1	0.6	0.6	0.5
PPM	0.6 ± 0.1	0.5	0.5	2.4
PoLO	0.8 ± 0.1	0.5	0.5	0.3
SSS	0.6 ± 0.1	0.5	0.5	0.8
PoPL	0.6 ± 0.0	0.4	0.3	0.0
MLO	0.3 ± 0.1	0.4	0.3	0.0
LLS	0.3 ± 0.0	0.3	0.3	1.6
MPL	0.3 ± 0.0	0.3	0.3	0.0
SSPo	0.7 ± 0.1	0.2	0.3	0.0
PoLS	0.3 ± 0.1	0.2	0.2	0.0
Other	0.4	1.9	1.4	1.2
Sum	100.0	100.0	100.0	100.0
Av. rel. err. % (n = 35)	31.4	—	10.1	28.9

[a]Abbreviations and column headings as in Table 1. Other abbreviations: M, myristic acid (14:0); Po, palmitoleic acid (16:1).

TABLE 8
Fatty Acid Percentage Composition of Randomized Lard TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	TG position 2
M	2.2	2.0	1.9	0.8	1.0
Po	4.9	2.7	2.7	1.1	2.5
P	30.4	29.9	29.8	29.7	29.5
Ln	0.0	0.3	0.0	0.0	0.3
L	7.6	7.9	8.0	7.8	8.9
O	37.1	40.6	40.9	44.0	40.7
S	17.7	16.7	16.6	16.6	17.1
Sum	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	19.0	—	1.0	21.7	

[a]Abbreviations as in Table 1 and column headings as in Table 4. Other abbreviations as in Table 7.

analysis of samples of known composition containing all species of interest. It was hoped that a complex synthetic mixture could be produced which, by nature of the method of its synthesis, would have a known composition, which could then be used as a calibration mixture. Toward this end, the mixture of 35 TG derived from five FA was produced. However, as the results in Table 3 showed, it quickly became apparent that there was sufficient concentration dependence in the response of the TG that such a universal calibration mixture was not adequate. Examination of the calibration curves confirmed that there was a noticeable change in slope of the points at lower concentration vs. those at higher concentration, which resulted in a concave appearance of the curves. Thus, while overall the calibration curves indicated linear responses over a broad concentration region, within different regions of the curves enough difference in slope was present to indicate that there was potential for problematic effects in calculation of percentage composition in mixtures containing TG present in disparate percentages.

Another method for production of a complex mixture of known composition was necessitated. Just as the composition of the synthetic mixture was given by the statistical distribution of the FA used for the synthesis, the composition of a completely randomized sample is given by the statistical distribution of its FA composition. Thus, the FA composition determined by calibrated GC–FID could be used to determine the composition of a completely randomized sample. This approach has the advantage that, if the randomized sample was used for calibration of a similar nonrandomized sample, the FA composition should be the same, so that the concentration differences between TG species arising from those FA in the two samples would not be great. However, unlike the synthetic mixture, for which there was no theoretical reason to believe that a random distribution was not achieved, a randomized fat or oil sample depends on dissociation and then reassociation of FA from/to the glycerol backbone, so independent steps had be taken to confirm that randomization occurred. Lipase hydrolysis has become a commonly used tool for determination of the positional distribution of FA within TG species, by differentiating between FA at the carbon 1,3 positions vs. the carbon 2 position. While not perfect, this approach is generally considered useful to indicate the degree of randomization achieved. For a completely randomized sample, the composition determined for the FA at carbon 2 equals the total FA composition. This is in distinct contrast to most natural fats and oils, which show distinct preferential placement of FA, depending on the type of fat or oil. The differences in FA placement in normal vs. randomized oils is seen by comparison of the lipase data for randomized vs. normal SBO in Tables 4 and 6, respectively. Normal SBO showed much less palmitic acid in the carbon 2 position vs. the total FA composition, but more linoleic acid in this position vs. the total FA composition. In contrast, the randomized sample showed much better agreement between the total FA

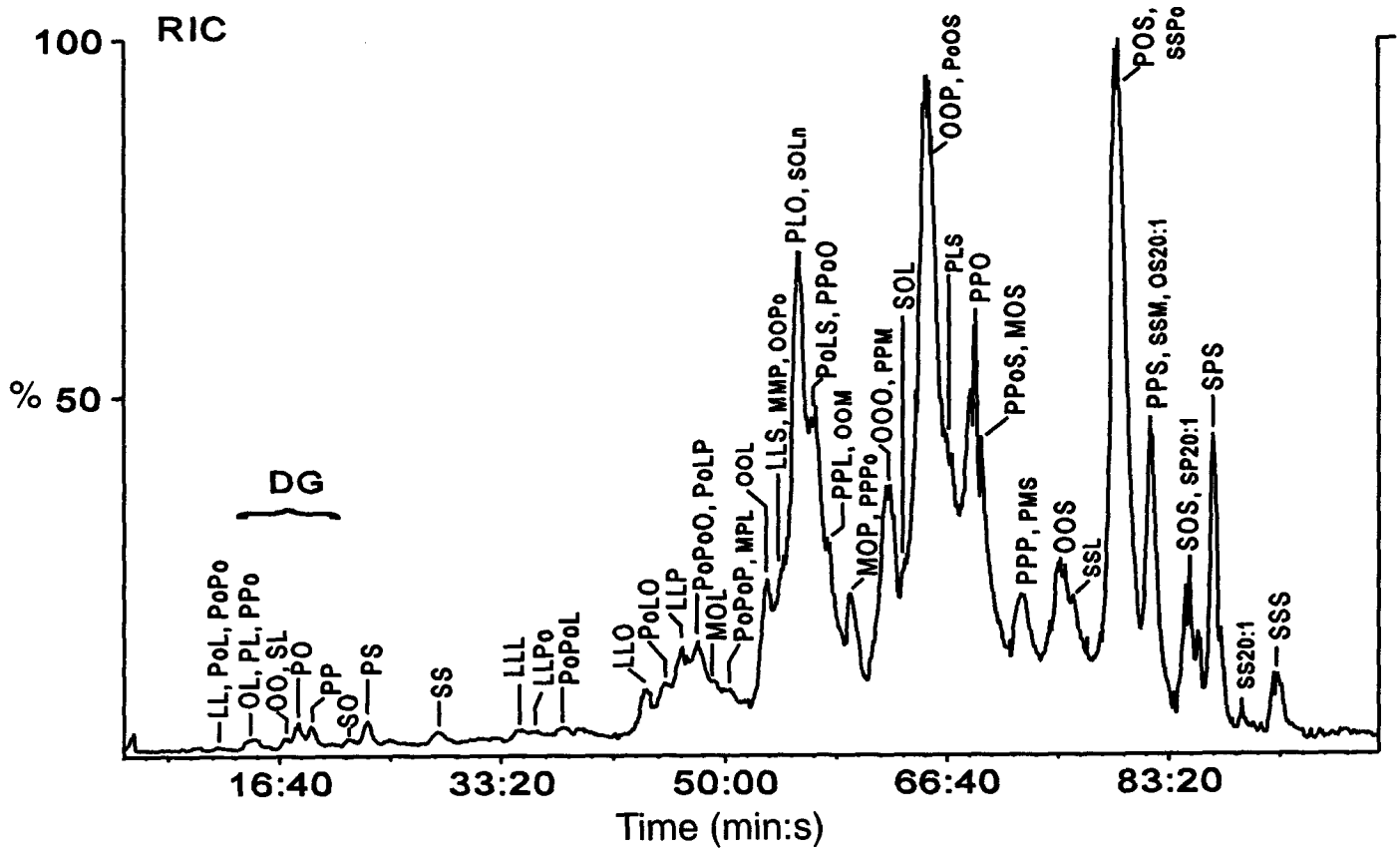

FIG. 10. Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry chromatogram of normal lard. Peak labels identify DG or triglyceride molecular species. See Figures 1, 2, 3, and 8 for abbreviations.

composition and the composition at carbon 2, though the agreement was not absolute, indicating that the randomization was not absolute. Opposite trends were observed in the lipase data for randomized vs. normal lard. Palmitic acid was preferentially incorporated into the 2-position of normal lard vs. randomized lard, shown in Tables 8 and 10, respectively, while less of oleic and linoleic acids appeared in the 2-position. More importantly, there was excellent agreement between the FA composition at the 2-position vs. the total FA composition, indicating a high degree of randomization.

The lipase data indicated that calculation of the TG composition of the randomized samples from the statistical distribution of the FA composition was expected to provide a good representation of the TG composition of the sample. Then, using the calculated TG composition to calculate response factors for each TG resulted in an adjusted TG composition equal to the random distribution. When the response factors thus derived were applied to the nonrandomized samples, good agreement was obtained between this method and another quantitation method, which used response factors based on FA. Importantly, the FA composition of the normal samples calculated from the TG composition obtained by application of the response factors calculated from the randomized samples showed excellent agreement with the FA composition obtained by calibrated GC–FID of the normal samples. The agreement with the GC–FID FA composition was better than that obtained from the TG composition determined by LC–FID, which was also in good agreement with the GC–FID results. However, the LC–FID detector did fail to identify numerous TG components. In the lard samples, most of these unidentified species contained myristic and palmitoleic acids, so these FA produced large relative error in the FA composition.

The response factors calculated using the synthetic mixture and the randomized samples all reflected the dependence of the qualitative behavior of the response factors on the fragmentation behavior of the TG, in combination with practical complications such as isotope peaks chromatographically coeluted with certain FA. The FA compositions calculated from the TG composition determined by APCI–MS also showed the same trends. Thus it seemed reasonable to use the FA composition for calculation of response factors to compensate for overrepresented and underrepresented FA. Several approaches were tried, but the best results came from simple multiplication of individual FA response factors together to produce TG response factors. The results obtained for all samples using this simple approach were in good agreement with calculated results, and the FA compositions calculated from the adjusted TG compositions were also in good agreement with the GC–FID results. Like the approach

TABLE 9
Percentage Composition of Normal Lard TG[a]

TG	Average raw MS % ($n = 6$)	GC–FID RF adjusted %	Randomized-lard-adjusted %	LC–FID %
OOP	17.1 ± 2.0	20.9	20.4	25.7
POS	16.7 ± 0.4	16.1	18.1	18.2
PLO	5.6 ± 0.5	6.9	6.8	7.5
PPO	6.2 ± 0.8	7.3	6.5	7.6
OOS	4.6 ± 0.5	4.6	5.1	7.2
OOO	3.8 ± 0.6	4.9	4.6	5.2
SSP	4.4 ± 0.4	3.4	3.9	2.9
PPS	4.5 ± 0.6	4.2	3.8	3.5
PLS	3.1 ± 0.3	3.0	3.2	5.0
OOL	2.7 ± 0.3	3.4	2.8	2.3
LOS	1.8 ± 0.1	1.8	2.3	1.0
SSO	2.5 ± 0.1	1.9	2.2	1.5
OOPo	2.5 ± 0.5	1.6	1.8	0.0
PoPO	2.8 ± 0.4	1.8	1.7	3.6
PPP	1.9 ± 0.3	2.2	1.6	1.2
PoOS	2.6 ± 0.2	1.4	1.5	0.0
OOM	1.0 ± 0.1	1.2	1.3	0.0
PPL	1.1 ± 0.2	1.4	1.2	1.0
MOS	0.9 ± 0.3	0.9	1.1	0.0
MPO	1.1 ± 0.2	1.3	1.0	0.0
SSS	1.2 ± 0.2	0.7	0.9	0.9
PoPS	1.5 ± 0.2	0.8	0.7	0.0
PoLO	1.1 ± 0.1	0.7	0.7	0.4
LLP	0.6 ± 0.1	0.8	0.6	0.8
LLO	0.8 ± 0.1	1.0	0.6	0.5
MPS	0.7 ± 0.2	0.6	0.6	0.0
MLO	0.4 ± 0.1	0.5	0.6	0.0
SSL	0.5 ± 0.1	0.4	0.4	0.0
PoPL	0.5 ± 0.0	0.3	0.3	0.0
PoLS	0.5 ± 0.1	0.2	0.3	0.0
PPM	0.3 ± 0.0	0.4	0.3	1.5
PPPo	0.6 ± 0.1	0.3	0.3	0.0
LLS	0.2 ± 0.0	0.2	0.3	1.5
MMO	0.4 ± 0.7	0.4	0.3	0.0
SSPo	0.7 ± 0.1	0.3	0.2	0.0
Other	3.0	2.1	1.9	1.0
Sum	100.0	100.0	100.0	100.0

[a]Abbreviations as in Table 1 and column heading as in Table 5. Other abbreviations as in Table 7.

TABLE 10
Fatty Acid Percentage Composition of Normal Lard TG[a]

FA	From raw MS TG	From calibrated GC–FID	From GC–FID-adjusted TG	From LC–FID TG	From random. SBO adjusted TG[b]	TG position 2
M	2.4	2.1	2.4	0.5	2.2	2.3
Po	5.1	2.8	2.9	1.4	2.8	3.9
P	28.8	30.3	30.1	31.8	29.0	57.3
Ln	<0.05	0.3	0.0	0.0	0.1	0.4
L	7.4	7.9	8.2	7.7	7.7	5.1
O	36.9	40.1	40.1	42.6	40.2	22.9
S	19.3	16.4	16.3	16.1	18.0	8.1
Sum	100.0	100.0	100.0	100.0	100.0	100.0
Avg. relative error %	22.2	—	3.9	24.0	3.8	

[a]Abbreviations as in Table 1 and column headings as in Table 4. Other abbreviations as in Table 7.

which used response factors calculated from randomized samples, this approach gave composition information for many species which were undetected by LC–FID. This approach had an advantage over the use of response factors derived from the randomized samples: it was equally applicable to randomized and to normal samples alike. The response factors were calculated based on the net over- or under-response of the FA, with no dependence on statistical distribution. Thus, whether a sample has been completely randomized or not was irrelevant, because the FA composition was determined using GC–FID. Response factors produced from the randomized mixtures were dependent on the statistical distribution of the FA, so lipase hydrolysis, or other method for confirmation of the randomization, was necessary.

Two methods for quantitation of TG composition using LC/APCI–MS data have been presented which provided good agreement to each other and to expected results. They both yielded FA compositions which were in good agreement with FA compositions obtained using GC–FID analysis. The choice of which method is most appropriate for a particular use depends on the goals of the total analytical scheme being employed. Simple analyses of fat and oil TG composition may make use of the simplicity of calculation of response factors with only GC–FID data required. On the other hand, some analyses, such as studies of genetically modified seed oils or dietary studies, often require the use of additional techniques, like lipase hydrolysis, as routine aspects of the analytical approach, so this information should be used, if available. Both of the methods described yielded results which showed less relative error than the commonly used quantitation method of LC–FID analysis. Although the FA compositions calculated from the TG compositions obtained using LC–FID were in good agreement with the FA compositions obtained by GC–FID (except for FA present at moderately low levels), the TG composition obtained by LC–FID contained numerous omissions of TG species which were conclusively identified using LC/APCI–MS. The two-dimensional nature of the FID, as well as ELSD and other detectors, imposes inherent limitations on their ability to fully characterize complex fat and oil samples. A simple and versatile analytical method like LC/APCI–MS makes mass spectrometric detection an attractive alternative for complete qualitative and quantitative characterization of complex TG mixtures.

REFERENCES

1. Shukla, V.K.S. (1988) Recent Advances in the High-Performance Liquid Chromatography of Lipids, *Prog. Lipid Res. 27*, 5–38.
2. Ruiz-Gutierrez, V., and Barron, L.J.R. (1995) Methods for the Analysis of Triacylglycerols, *J. Chromatogr. B 671*, 133–168.
3. Myher, J.J., and Kuksis, A. (1995) General Strategies in Chromatographic Analysis of Lipids, *J. Chromatogr. B 671*, 3–33.
4. Byrdwell, W.C., and Emken, E.A. (1995) Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization Mass Spectrometry, *Lipids 30*,173–175.

5. Neff, W.E., and Byrdwell, W.C. (1995) Soybean Oil Triacylglycerol Analysis by Reverse-Phase High-Performance Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry, *J. Am. Oil Chem. Soc. 72*, 1185–1191.
6. Adlof, R.O., Miller, W.R., and Emken, E.A. (1978) Synthesis of *cis* and *trans* Methyl 8- and 13- Octadecenoate-d_2 and d_4 Isomers, *J. Label. Comp. Radiopharm. 15*, 625.
7. Osborn, J.A., Jardine, F.H., Young, J.F., and Wilkinson, G. (1966) The Preparation and Properties of *Tris*(triphenylphosphine)halo-genorhodium(I) and Some Reactions Thereof Including Catalytic Homogeneous Hydrogenation of Olefins and Acetylenes and Their Derivatives, *J. Chem. Soc. (A)*, 1711–1732.
8. Wheeler, D.H., Reimenschneider, R.W., and Sando, C.E. (1940) Preparation, Properties, and Thiocyanogen Absorbtion of Triolein and Trilinolein, *J. Biol. Chem. 132*, 687–699.
9. List, G.R., Emken, E.A., Kwolek, W.F., and Simpson, T.D. (1977) "Zero *trans*" Margarines: Preparation, Structure, and Properties of Interesterified Soybean Oil–Soy Trisaturate Blends, *J. Am. Oil Chem. Soc. 54*, 408–413.
10. Glass, R.L. (1969) Preparation of Milk Fat Methyl Esters by Alcoholysis in an Essentially Nonalcoholic Solution, *J. Dairy Sci. 52*, 1289–1290.
11. Zeitoun, M.A.M., Neff, W.E., Selke, E., and Mounts, T.L. (1991) Analyses of Vegetable Oil Triglyceride Molecular Species by Reverse-Phase High-Performance Liquid Chromatography, *J. Liquid Chromatogr. 14*, 2685–2698.
12. Neff, W.E., Zeitoun, M.A.M., and Weisleder, D. (1992) Resolution of Lipolysis Mixtures from Soybean Oil by a Solid-Phase Extraction Procedure, *J. Chromatogr. 589*, 353–357.
13. Daubert, B.F. (1949) The Composition of Fats, *J. Am. Oil Chem. Soc. 26*, 556–558.
14. Bailey, A.E., and Swern, D. (1964) *Bailey's Industrial Oil and Fat Products*, 3rd edn., pp. 965–968, John Wiley & Sons, New York.
15. Mares, P., Rezanka, T., and Novak, M. (1991) Analysis of Human Blood Plasma Triacylglycerols Using Capillary Gas Chromatography, Silver Ion Thin-Layer Chromatographic Fractionation and Desorption Chemical Ionization Mass Spectrometry, *J. Chromatogr. 568*, 1–10.
16. Rezanka, T., and Mares, P. (1991) Determination of Plant Triacylglycerols Using Capillary Gas Chromatography, High-Performance Liquid Chromatography and Mass Spectrometry, *J. Chromatogr. 542*, 145–159.
17. Myher, J.J., Kuksis, A., Marai, L., and Manganaro, F. (1984) Quantitation of Natural Triacylglycerols by Reverse-Phase Liquid Chromatography with Direct Liquid Inlet Mass Spectrometry, *J. Chromatogr. 283*, 289–301.
18. Kuksis, A., Marai, L., and Myher, J.J. (1983) Strategy of Glycerolipid Separation and Quantitation by Complementary Analytical Techniques, *J. Chromatogr. 273*, 43–66.
19. Hites, R.A. (1970) Quantitative Analysis of Triglyceride Mixtures by Mass Spectrometry, *Anal. Chem. 42*, 1736–1740.
20. Phillips, F.C., Erdahl, W.L., Schmit, J.A., and Privett, O.S. (1984) Quantitative Analysis of Triglyceride Species of Vegetable Oils by High Performance Liquid Chromatography *via* a Flame-Ionization Detector, *Lipids 19*, 880–887.

[Received November 27, 1995, and in final revised form June 19, 1996; Revision accepted July 5, 1996]